

# Anti-oxidative functions of *mt2* and *smtB* mRNA expression in the gills and brain of zebrafish (*Danio rerio*) upon cadmium exposure

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**Abstract** There were not any past studies about metallothionein isoforms (*smtB* and *mt2*) having anti-oxidative functions on zebrafish after  $\text{Cd}^{2+}$  exposure. On the other hand, the anti-oxidative enzymatic factors such as superoxide dismutase (*sod*), glutathione peroxidase (*gpx1a*), and catalase (*cat*) are used as references to investigate whether the *smtB* and *mt2* have anti-oxidative responses on the gills and brain of zebrafish after 1–6 h of 0 and 1.78  $\mu\text{M}$   $\text{Cd}^{2+}$  exposure. The anti-oxidative system such as *sod*, *cat*, and *gpx1a* mRNA expressions demonstrated a cascade response upon  $\text{Cd}^{2+}$ -induced oxidative stress in the present study. Interestingly, the *smtB* mRNA expression levels increased by 3.2- to 6.1-fold, and *mt2* raised by 4.1- to 11.3-fold in gills at 1 and 3 h after exposure to  $\text{Cd}^{2+}$ , respectively. On the other hand, the *smtB* mRNA levels increased by 10.6- to 58.6-fold, but *mt2* mRNA levels increased by 2.3- to 11.1-fold in brain at 1 and 3 h after exposure to  $\text{Cd}^{2+}$ , respectively. In addition, both tissues showed increased apoptosis levels at 3 h, and recovery after 6 h of  $\text{Cd}^{2+}$  exposure. From the results, we suggest that both *mt2* and *smtB* play a role in anti-oxidation responses within 6 h after exposure to  $\text{Cd}^{2+}$ . In conclusion, the *smtB* mRNA levels have a higher response than *mt2* in the brain, but both mRNA expressions appear to have a similar

pattern in the gill. We suggest that *smtB* plays an important role to defend oxidative stress in the brain of adult zebrafish upon acute  $\text{Cd}^{2+}$  exposure.

**Keywords** Apoptosis · Metallothionein · Reactive oxygen species · Zebrafish · Cadmium

## Introduction

Metallothioneins (MTs) have been found around 60 years ago, and its physiological functions have also evolved, including the detoxification of heavy metals such as cadmium (Cd) and mercury, and the regulation of some essential metals such as copper and zinc in teleosts (Roesijadi 1992). In addition, MT may be induced in response to oxidative stress and may function to protect tissues from oxidative damage (Sato and Bremner 1993). In zebrafish (*Danio rerio*) larvae, the genes *mt2* and *smtB* first appeared in 6 hpf (hours of post-fertilization) and 12 hpf of the embryonic stage, respectively (Wu et al. 2008a). Our previous studies found that both genes appeared to have different functions between larvae and adult fish. For example, the *mt2* signals appeared upon ionic cadmium ( $\text{Cd}^{2+}$ ) exposure and cold shock, but *smtB* signals were not found under the same treatment conditions by whole-mount in situ hybridization in developmental larvae (Wu et al. 2008a). However, the *mt2* and *smtB* mRNA levels were, respectively,

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increased by 4- and 3.3-fold in the brain of adult fish after cold shock (Wu et al. 2015). The *mt2* mRNA expression noticeably increased after exposure to waterborne metals, but the phenomenon was not copied for *smtB* in the ovaries of female zebrafish (Wu et al. 2012). On the other hand, it was interesting to find that the *smtB* mRNA showed a tissue-specific expression, and it indicated that the brain contained 2- to 3-fold more of the *smtB* mRNA expression as compared to other tissues (e.g., gill, liver, and intestine). However, tissue-specific expressions relating to *mt2* mRNA cannot be found in adult zebrafish (Wu et al. 2008a). Therefore, the present study was to study the physiological roles of *smtB* and *mt2* in the brain after  $\text{Cd}^{2+}$  exposure.

The brain may be the most sensitive organ to environmental fluctuations, since most of the physiological acclimation responses are initiated by the central nervous system (Crawshaw et al. 1985). Therefore, the brain is a highly energy-consuming organ, and the high metabolic rate of brain cells implies a high production of ROS (Tseng et al. 2011). Thus, the brain requires a powerful anti-oxidative system in order to avoid oxidative stress and keep cellular redox state in balance. On the other hand, the gills are the first organ to be affected by exposure to waterborne trace elements (Taylor et al. 1996), and our previous study has found that the gills seem to have a powerful repairing system after exposure to metal (Wu et al. 2008b). Therefore, it is interesting to compare with the anti-oxidative function of metallothionein isoforms between the two organs. Our previous study found that the RT-PCR products of relative expressions of MT mRNA and  $\beta$ -actin of *mt2* were down-regulated in the brain, and *smtB* was not induced in gills but can be induced in the brain by  $\text{Cd}^{2+}$  on mature zebrafish (Wu et al. 2008a). These results raise questions regarding the physiological functions of *smtB* and *mt2* in the brain and gills of adult zebrafish after  $\text{Cd}^{2+}$  exposure. A particular focus is placed on the anti-oxidative functions.

Cadmium is a nonessential and toxic metal that can be found at elevated levels in water and soil. Cuypers et al. (2010) reported that  $\text{Cd}^{2+}$  could indirectly generate free radicals.  $\text{Cd}^{2+}$  damages cells while simultaneously causing the formation of reactive oxygen species (ROS) (Leonard et al. 2004). Thus, it is suspected that the exposure of aquatic animals to  $\text{Cd}^{2+}$  causes biochemical or physiological changes

that can protect organisms from  $\text{Cd}^{2+}$  toxicity. In addition, antioxidant responses will be induced by chemical and physical stresses, even for limited durations. However, the action of  $\text{Cd}^{2+}$  toxicity can potentially disrupt the antioxidant potential and cause oxidative stress (Bagnyukova et al. 2007). Like many other vertebrates, fish use antioxidant defense mechanisms to reduce damage from oxidative stress. There were differential types of antioxidant defense mechanisms which included anti-oxidant molecules, such as glutathione (GSH), ascorbic acid, tocopherol, and carotenoids (Alvarez et al. 2005), and antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (Valavanidis et al. 2006). Particularly, several studies have examined the role of non-enzyme antioxidants such as metallothioneins (MTs) (Shimoda et al. 2003; Chen and Maret 2001). MTs are a small family of thiol-rich proteins that exhibit antioxidant activities and, together with GSH, are important in the regulation of redox homeostasis and protection against superoxide and hydroxyl radicals (HO.) (Chen and Maret 2001). Recently, it has been demonstrated that both genes (*mt2* and *smtB*) appear to have an anti-oxidative function upon cold shock (Wu et al. 2015). However, it has not yet been confirmed if *mt2* and *smtB* might have different anti-oxidative roles between tissues upon  $\text{Cd}^{2+}$  exposure.

Oxidative stress generated by  $\text{Cd}^{2+}$  and other stresses can cause DNA damage. The oxidation of DNA bases can be due to a HO. radical attack on the pyrimidines aromatic cycles. Lipid peroxidation is also a source of endogenous DNA damage via malondialdehyde (MDA) and 4-hydroxy-2-Nonenal (HNE) which can react with DNA bases. ROS reaction to proteins can also produce new radicals that can react with DNA. These examples of DNA damage are induced by lipids and protein oxidation products, which has shown to cause the indirect genotoxic effects of oxidative stress. In other words, a cascade response of oxidative stress, which was induced by ROS production, oxidative DNA damage, and lipid and protein oxidation, resulted in short-term cell apoptosis (Choi et al. 2007). However, during short periods of stress, antioxidant molecules, antioxidant enzymes, and proteins protect the organism to keep off oxidative stress, in which the antioxidant defense system, e.g., *sod*, *cat*, *gpx1a*, and *mt2* mRNA expressions, was all up-regulated after 1 h of cold shock, and

the *smtB* mRNA gene expression was up-regulated after 6 h of cold shock in zebrafish (Wu et al. 2015). Thus, the present study seeks to confirm that *smtB* and *mt2* play a physiological anti-oxidative role following the up-regulation of ROS and LPO levels, *sod*, *gpx1a*, and *cat* levels within 6 h following Cd<sup>2+</sup> exposure. In addition, levels of cells apoptosis in the brain and gills upon Cd<sup>2+</sup> exposure were observed in adult zebrafish.

## Materials and methods

### Animals and Cd<sup>2+</sup> exposure

Sexually mature zebrafish (*D. rerio*) of both sexes were obtained from the Taiwan Zebrafish Core Facility (TZCF, Miaoli, Taiwan). The fish were kept in an aquarium supplied with circulating, dechlorinated, aerated local tap water at 28 °C with a 14 h:10 h light and dark photoperiod. Fish were fed both *Daphnia pulex* and *Artemia franciscana* prior to the experiments. Following conditions from our previous study, a concentration of 1.78 µM Cd<sup>2+</sup> was used. The experimental medium was prepared with 1.78 µM Cd<sup>2+</sup> in dechlorinated, circulated, aerated local tap water. The water quality parameters included a total hardness of 146.6 ± 5.6 mg/L, Na<sup>+</sup> of 35.6 ± 0.3 mg/L, K<sup>+</sup> of 3.3 ± 0.1 mg/L, Ca<sup>2+</sup> of 30 ± 2.3 mg/L, O<sub>2</sub> of 5–7 mg/L, and pH of 8.2 ± 0.3; Cd<sup>2+</sup> concentration was <1 µg/L. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC Approval No. 101003).

### Experimental settings and sampling

#### Experiment 1

Experiment 1: Eight test tanks were used in the present study, and five male zebrafish were used in each tank to prevent inconsistent gender distributions from causing different physiological responses upon Cd<sup>2+</sup> exposure (Sellin et al. 2007). Four tanks of control groups contained 0 µM Cd<sup>2+</sup> water, and four tanks of treatment groups contained 1.78 µM Cd<sup>2+</sup> water. Experiments ran for 6 h with sampling at 0, 1, 3, and 6 h after Cd<sup>2+</sup> exposure (*n* = 5). Groups of five control zebrafish were sampled at each time interval. The fish were immediately anesthetized with 50 mg/L

MS222 (Ethyl 3-aminobenzoate methanesulfonate salt; Sigma) after 0, 1, 3, and 6 h of Cd<sup>2+</sup> exposure, and the gills and brain were excised from the body to determine *cat*, *sod*, *gpx1a*, *mt2*, and *smtB* mRNA expression after each sampling time.

#### Experiment 2

Apoptosis cells were measured with TUNEL assay. Four test tanks were used with 1 tank containing 0 µM Cd<sup>2+</sup> water (negative and positive control group) and 3 other tanks containing 1.78 µM Cd<sup>2+</sup> water (treatment group). The fish were taken from the tanks, and the brain and gills were excised after 0, 3, and 6 h of various doses of Cd<sup>2+</sup> exposure. All experiments were repeated 3 times.

#### Preparation mRNA and cDNA synthesis

To prepare total RNA, 5 mg of gills and brain tissues was homogenized in 800 µL of Trizol reagents (Invitrogen, Carlsbad, CA, USA) and treated according to the manufacturer's protocols. The amount and quality of total RNA were determined by measuring the absorbance at 260/280 nm with a spectrophotometer (NanoDrop ND-1000, DE, USA) and analyzed using RNA-denatured gels. The total amount of RNA was subsequently extracted using a QuickPrep Micro mRNA purification kit (Amersham Pharmacia, Piscataway, NJ, USA). Finally, the mRNA pellets were precipitated with 0.1 mg glycogen, 1/10 the volume of 3 mM NaOAc, and 95 % ethanol, stored at –20 °C for 10 min, then centrifuged at 13,500×*g* for 15 min in order to do cDNA synthesis. A first-strand cDNA library for both tissues was then constructed according to the protocol of the SMART<sup>TM</sup> cDNA Library Construction Kit (Clontech, USA) and stored at –20 °C until QPCR analysis.

#### Quantitative real-time PCR of *sod*, *cat*, *gpx1a*, *mt2*, *smtB* mRNA and β-actin

Quantitative real-time PCR (qPCR) was carried out using a SYBR Green dye-based assay (Qiagen, Hilden, Germany) with an ABI Prism 7500 Sequence Detection System (Perkin-Elmer, Applied Biosystems, Wellesley, MA, USA) according to the manufacturer's instructions. We followed Choi et al. (2010) in selecting primers targeting *SOD*, *CAT*, and *GPx*,

and Wu et al. (2012) in selecting primers targeting *smtB* and *mt2*. The internal control gene  $\beta$ -actin was designed using the Primer Express 2.0 software (Applied Biosystems). In each assay, 25 ng cDNA was amplified in a 25  $\mu$ L reaction containing 12.5  $\mu$ L of Platinum SYBR Green *q*PCR SuperMix-UDG (Invitrogen, California), 0.5  $\mu$ L (200 nM) of forward and reverse primers, and nuclease free water. The primers used for the consensus of the selected genes and internal control gene ( $\beta$ -actin) are listed in the table below.

| Gene               | Forward primer (5′–3′)            | Reverse primer (5′–3′)         |
|--------------------|-----------------------------------|--------------------------------|
| <i>SOD</i>         | GGC CAA CCG ATA<br>GTG TTA GA     | CCA GCG TTG CCA<br>GTT TTT AG  |
| <i>Cat</i>         | AGG GCA ACT GGG<br>ATC TTA CA     | TTT ATG GGA CCA<br>GAC CTT GG  |
| <i>GPx1a</i>       | ACC TGT CCG CGA<br>AAC TAT TG     | TGA CTG TTG TGC<br>CTC AAA GC  |
| <i>sMT-B</i>       | TGC TCC AAA TCT GGA<br>TCT TG     | GCA GTC CTT CTT<br>GCC CTT AC  |
| <i>MT-2</i>        | AGA CTG GAA CTT<br>GCA ACT GTG GT | CAG CTG GAG CCA<br>CAG GAA TT  |
| $\beta$ -<br>actin | CGG AAT CCA CCA<br>AAC CAC CTA    | ATC TCC TTC TGC<br>ATC CTG TGA |

All *q*PCRs were performed as follows: 1 cycle at 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and 58–60 °C for 15 s (with times depending on the standard annealing temperature of all primers). PCR products were subjected to a melting curve analysis, and representative samples were electrophoresed to verify that only a single product was present. Control reactions were conducted with sterile water to determine levels of background and genomic DNA contamination. The standard curve of each gene was confirmed to be in a linear range with  $\beta$ -actin as an internal control. The relative quantity formulae are as follows:

$$\Delta Ct_{(\text{treatment})} = \text{target gene Ct} - \beta\text{-actin Ct}$$

$$\Delta Ct_{(\text{control})} = \text{target gene Ct} - \beta\text{-actin Ct}$$

$$\Delta\Delta Ct = \Delta Ct_{(\text{treatment})} - \Delta Ct_{(\text{control})}$$

$$\text{Relative Quantity} = 2^{-\Delta\Delta Ct}$$

Within *q*PCR reactions, triplicate reactions were performed for each sample, including  $\beta$ -actin (internal

control) and target genes. To ensure data precision, *q*PCR data were accepted only when the variation Ct values were less than 1 in triplicate reactions of each gene within the whole set of experiments.

#### Lipid hydroperoxide (LPO) assay and reactive oxygen species (ROS) assay

LPO assays were conducted using a method modified from Porter et al. (1995), while the ROS assays were conducted using a method modified from Imada et al. (1999). The 5 mg of gills and brain tissues was individually homogenized in a homogenization buffer either with 500  $\mu$ L of Extract R-Saturated methanol solution for the LPO assay or with 200  $\mu$ L of L-012 (8-Amino-5-chloror-7-phenylpyrido(3,4-*d*)pyridazine-1,4-(2H,3H) dione sodium salt) (Wako, Japan) for the ROS assay. Briefly, L-012 was dissolved into ddH<sub>2</sub>O to a final concentration at 20 mM, and then diluted with an appropriate buffer [50 mM Tris–HCl (pH 7.5)] to be an assay that has a concentration of 4  $\mu$ M for O<sub>2</sub><sup>•−</sup> radical measurements. The homogenates were incubated with the 4  $\mu$ M of L-012 reaction medium for 10 min under a dark space, and then centrifuged at 12,000 $\times$ g for 3 min, after which 100  $\mu$ L of supernatants was used to measure the relative light units (RLU) with a luminescence instrument (Berthold, Lumat LB9507, Germany) (Imada et al. 1999).

#### Cell death detection

Cell death was detected using TUNEL assays to label DNA strand breaks. The TUNEL assay was performed on 5- $\mu$ m sections and reacted with an ApopTag<sup>®</sup> - Peroxidase In Situ Apoptosis Detection Kit S7100 (Millipore, USA). To remove the paraffin, the sections were washed in three changes of xylene over a period of 5 min each time and were then immersed in two changes of absolute ethanol. They were then washed twice in ethanol (95 and 70 % ethanol) for 5 min each, followed by rinsing with 0.5 % Triton X-100 (Triton X-100, JT Baker, USA) for 10 min, and finally twice with clean water for 2 min each. The sections were then immersed in 0.01 M PBS (Na<sub>2</sub>HPO<sub>4</sub>, NaCl, KCl, KH<sub>2</sub>PO<sub>4</sub>, and H<sub>2</sub>O, BSA 1 %; pH 7.4) buffer containing 3 % H<sub>2</sub>O<sub>2</sub> (30 % H<sub>2</sub>O<sub>2</sub>, Sigma, USA) for 5 min. The specimens were then rinsed with 65  $\mu$ L of equilibration buffer and incubated for at least 10 s at room temperature. Then 55  $\mu$ L of TdT enzyme

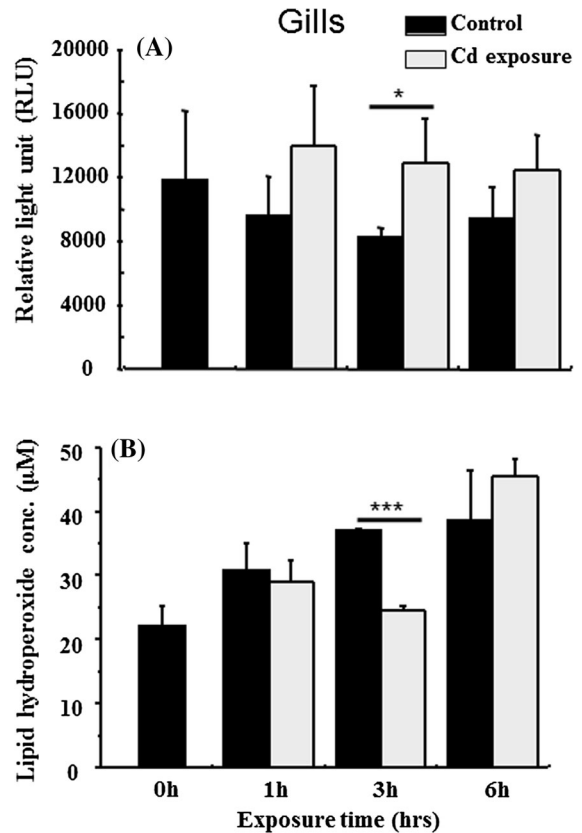
(reaction buffer: TdT enzyme = 7:3) was administered by pipette, followed by 1 h incubation in a humidified chamber at 37 °C. The slide was placed in a working strength stop/wash buffer, agitated for 15 s, and incubated for 10 min at room temperature. The slide was washed in three changes of PBS for 1 min each time, reacted with an anti-digoxigenin conjugate solution on the slide, and incubated in a humidified chamber for 30 min at room temperature. The slide was then washed in four changes of 0.01 M PBS before being completely covered in working strength peroxidase substrate (DBA dilution buffer: DBA substrate = 50:1) for 5 min at room temperature. Finally, the slide was stained with hematoxylin and covered with ClearMount (Invitrogen, USA, California). The positive control used DNase I (1U/μl) to induce DNA breakage in the initial step, and then followed the same steps described above. The positive control sample was treated with DNase solution (1U/μl) for 5 min at room temperature, while negative control staining was performed without active TdT, but was incubated with proteinase K digestion to control for the nonspecific incorporation of nucleotides or for the nonspecific binding of enzyme conjugates.

### Statistical analysis

Values are presented as the mean ± SD ( $n = 5$ ). Control and treatment groups at the same time points were compared with Student's *t* test with  $p < 0.05$  being accepted as a significant difference.

### Results

In the gills, the ROS significantly increased, but the LPO levels decreased significantly after exposure to Cd<sup>2+</sup> for 3 h as compared to the control group (Fig. 1). The *sod* and *cat* mRNA expressions were significantly up-regulated after the 1st h of Cd<sup>2+</sup> exposure as compared to the control group at the same treatment time, but both mRNA expressions recovered quickly. In addition, *gpx1a* mRNA levels significantly increased after Cd<sup>2+</sup> exposure for 3 h. Three anti-oxidative genes (*sod*, *cat*, and *gpx1a*) nearly recovered to their initial levels after exposure to Cd<sup>2+</sup> for 6 h (Fig. 2).

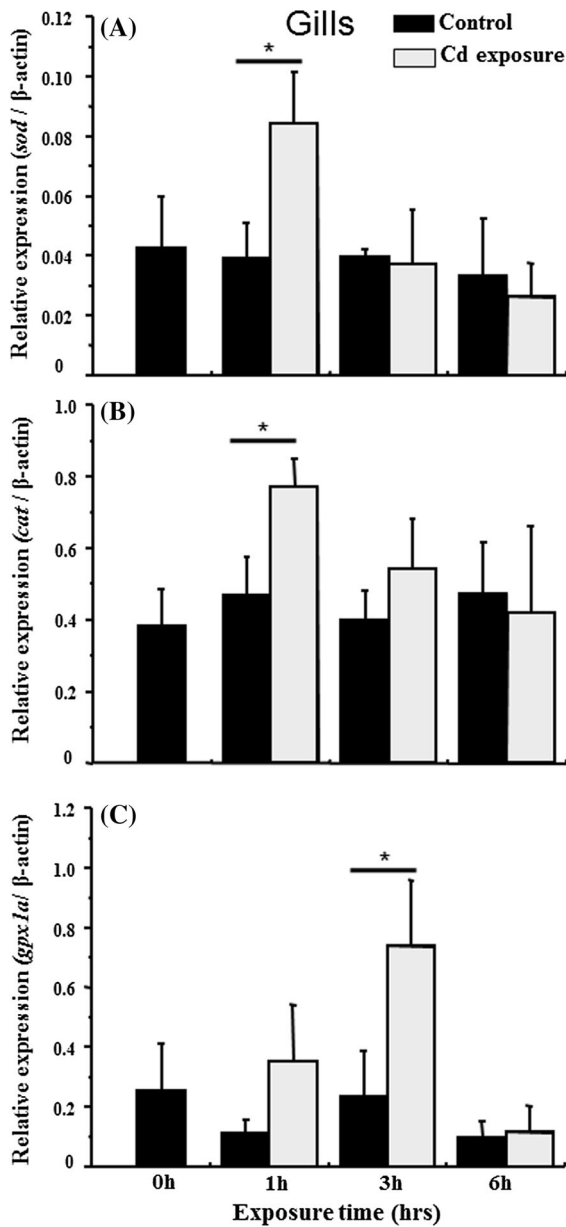


**Fig. 1** ROS (a) and LPO (b) levels in the gills after 0, 1, 3, and 6 h of exposure to 1.78 μM Cd<sup>2+</sup> (treatment), with 0 being the control. Data are given as mean ± SD ( $n = 5$ ), and data were analyzed by Student's *t* test to compare the control and treatment groups at the same treatment time (\* $p < 0.05$ ; \*\*\* $p < 0.001$ )

In the brain, the ROS increased significantly within 6 h after Cd<sup>2+</sup> exposure. However, LPO levels did not change significantly following Cd<sup>2+</sup> exposure (Fig. 3). *SOD* levels were not significantly changed after exposure to Cd<sup>2+</sup>, but *CAT* and *GPx* levels were significantly heightened at 1–3 h after Cd<sup>2+</sup> exposure (Fig. 4).

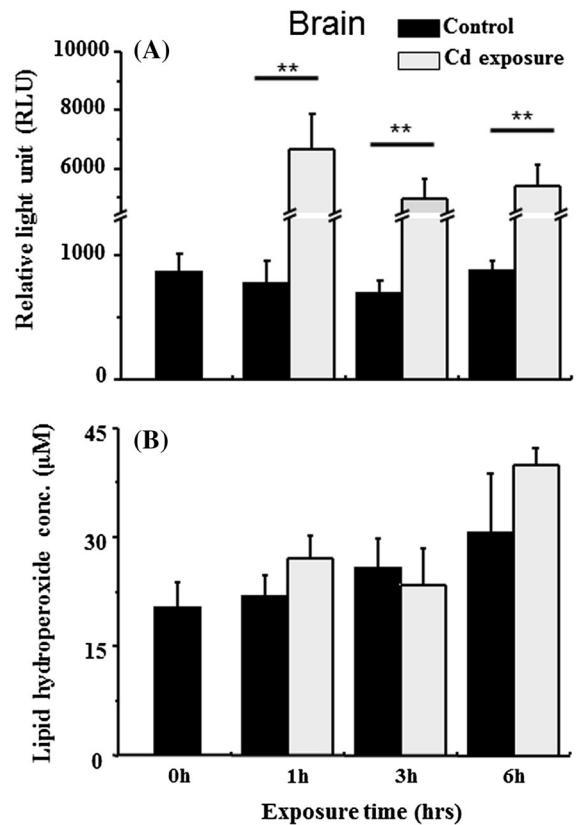
The *mt2* and *smtB* gene expressions in the gills increased significantly (4.1- to 11.3-fold and 3.2- to 6.1-fold, respectively) within 3 h after Cd<sup>2+</sup> exposure. In addition, *mt2* mRNA expression in the gills still exceeds the control 6 h after Cd<sup>2+</sup> exposure (Fig. 5). In the brain, *mt2* and *smtB* mRNA levels followed a similar pattern, respectively, increasing 2.3- and 10.6-fold after exposure to Cd<sup>2+</sup> for 1 h and 11.1- and 58.6-fold after exposure to Cd<sup>2+</sup> for 3 h. However, both genes' mRNA levels recovered 6 h after Cd<sup>2+</sup>





**Fig. 2** Changes of *sod* (a), *cat* (b), and *gp1a* (c) mRNA relative expressions in gills after 0, 1, 3, and 6 h of exposure to 1.78  $\mu\text{M}$   $\text{Cd}^{2+}$  (treatment), with 0 being the control. Data are given as mean  $\pm$  SD ( $n = 5$ ), and data were analyzed by Student's *t* test to compare the control and treatment groups at the same treatment time ( $*p < 0.05$ )

exposure (Fig. 6). In the brain and gills, the levels of MT genes (*mt2* and *smtB*) both showed a time-dependent increase after  $\text{Cd}^{2+}$  exposure for 1 and 3 h (Figs. 5, 6).

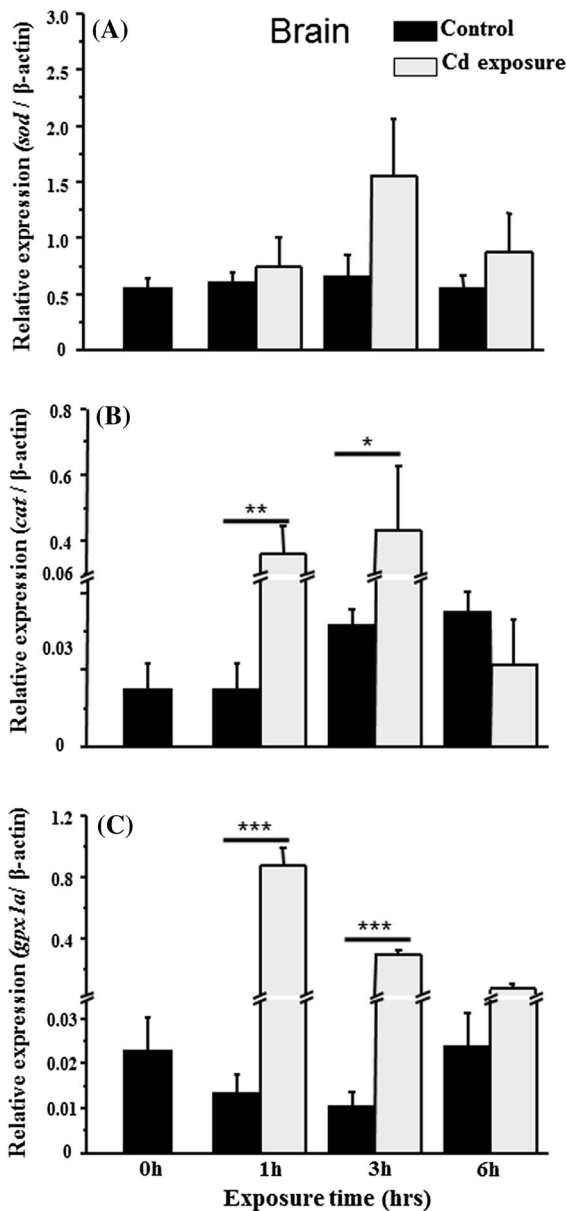


**Fig. 3** ROS (a) and LPO (b) levels in the brain after 0, 1, 3, and 6 h of exposure to 1.78  $\mu\text{M}$   $\text{Cd}^{2+}$  (treatment), with 0 being the control. Data are given as mean  $\pm$  SD ( $n = 5$ ), and data were analyzed by Student's *t* test to compare the control and treatment groups at the same treatment time ( $**p < 0.01$ )

No mortality was observed following  $\text{Cd}^{2+}$  exposure. The highest levels of cell apoptosis signals appeared after exposure to  $\text{Cd}^{2+}$  for 3 h, and recovery took place during a period of 3–6 h after 1.78  $\mu\text{M}$  of  $\text{Cd}^{2+}$  exposure in the gills (Fig. 7). Similar results were found in the brain, where the cell apoptosis reached its highest level 3 h after  $\text{Cd}^{2+}$  exposure, followed by recovery after 6 h (Fig. 8).

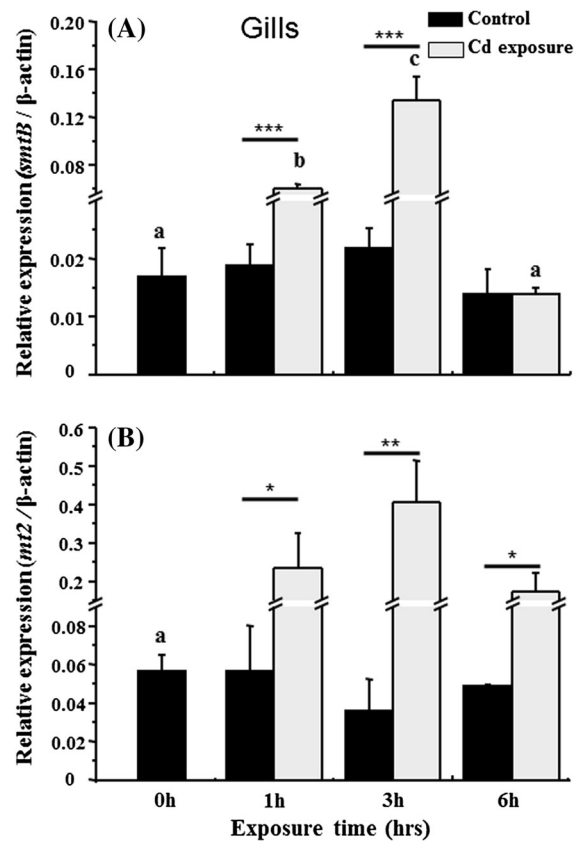
## Discussion

Previous studies have reported that heavy metals induce ROS in fish, and others have examined antioxidant gene expressions to decrease oxidative damage (Firat et al. 2009; Cao et al. 2010). Such defense mechanisms have been proposed as



**Fig. 4** Changes of *sod* (a), *cat* (b), and *gpx1a* (c) mRNA relative expressions in the brain after 0, 1, 3, and 6 h of exposure to 1.78 μM Cd<sup>2+</sup> (treatment), with 0 being the control. Data are given as mean ± SD ( $n = 5$ ), and data were analyzed by Student's *t* test to compare the control and treatment groups at the same treatment time ( $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ )

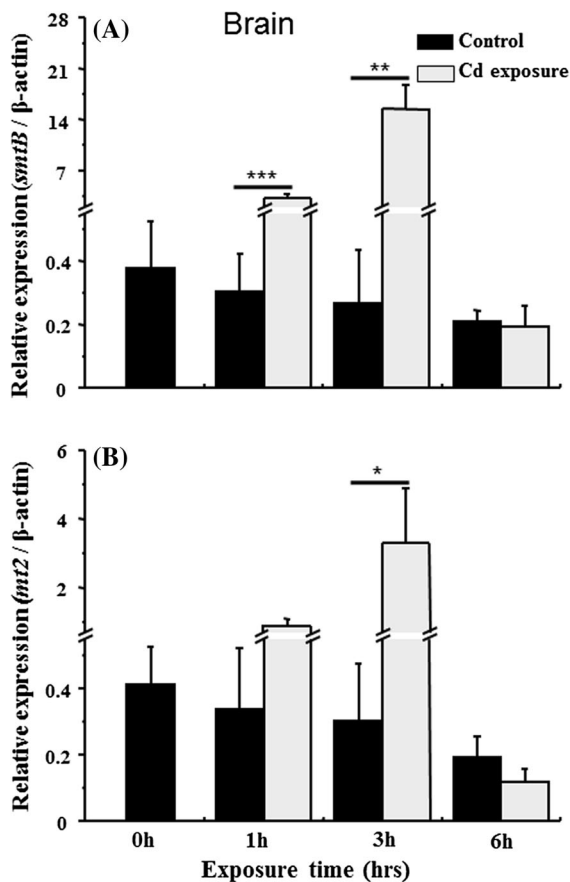
biomarkers following pro-oxidant challenge (Regoli et al. 2002). In addition, many studies have sought to monitor environmental pollution by tracking an organism's antioxidant biomarkers. For example, the *GPX* gene has emerged as a basic research tool for



**Fig. 5** Changes of *smtB* (a) and *mt2* (b) levels in gills after 0, 1, 3, and 6 h of exposure to 1.78 μM Cd<sup>2+</sup> (treatment), with 0 being the control. Data are given as mean ± SD ( $n = 5$ ), and data were analyzed by Student's *t* test to compare the control and treatment groups at the same treatment time ( $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ )

studying antioxidants and is used as an indicator for measuring environmental pollution or stress in fish (Choi et al. 2007). The present study found that Cd<sup>2+</sup> exposure exerted a range of impact such as cell apoptosis and the increase in mRNA expressions of antioxidant enzyme genes in the brain and gills of adult zebrafish.

Exposure to Cd<sup>2+</sup> has been found to cause a time- and dose-dependent increase in Cd<sup>2+</sup> contents in various fish species (Wu et al. 2006; Firat et al. 2009). Accumulation of Cd<sup>2+</sup> in fish could stimulate the production of ROS, which then reacts with susceptible biological macromolecules, resulting in changes to LPO and antioxidant defenses (Firat et al. 2009). In the present study, ROS levels of gills did not significantly change over various exposure times, but were elevated



**Fig. 6** Changes of *smtB* (a) and *mt2* (b) levels in the brain after 0, 1, 3, and 6 h of exposure to 1.78  $\mu\text{M}$   $\text{Cd}^{2+}$  (treatment), with 0 being the control. Data are given as mean  $\pm$  SD ( $n = 5$ ), and data were analyzed by Student's *t* test to compare the control and treatment groups at the same treatment time (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ )

in the treatment group as compared to the control group 3 h after  $\text{Cd}^{2+}$  exposure (Fig. 1a). However, results in the brain were significantly different, with ROS contents increasing significantly during 1–6 h after  $\text{Cd}^{2+}$  exposure, but no change was observed in the LPO levels upon  $\text{Cd}^{2+}$  exposure (Fig. 3). Increased ROS levels as a result of  $\text{Cd}^{2+}$  exposure probably produce antioxidant enzymes involved in protecting against oxidative stress. Increased LPO levels in fish following  $\text{Cd}^{2+}$  exposure are usually due to oxidative stress (Cao et al. 2010). Thus, the present study showed that the LPO level did not change in the brain even when the ROS were increased during exposure of  $\text{Cd}^{2+}$  for 1–6 h. These results were to prove that the brain has a powerful anti-oxidative

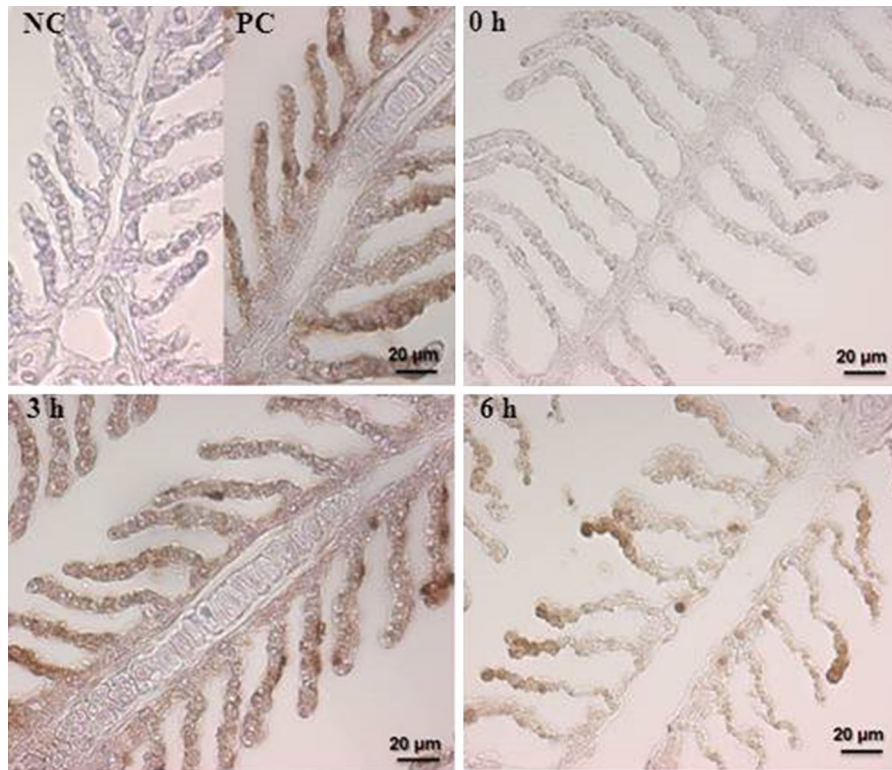
system under  $\text{Cd}^{2+}$  exposure. On the other hand, ROS level significantly increased around 1.5-fold and LPO levels on the treatment group was lower than the control following 3 h of  $\text{Cd}^{2+}$  exposure in the gills. The LPO level seems to be either a compensative physiological response or a yet identified reason, which could be investigated in the future.

The present study measured the mRNA expression of these antioxidants with real-time RT-PCR (*qPCR*) and normalization by  $\beta$ -actin. In fact,  $\beta$ -actin has been the most commonly used reference gene for normalizing *qRT-PCR* data in zebrafish (Tang et al. 2007). In addition, our previous study has reported that the zebrafish  $\beta$ -actin gene appeared suitable as a reference gene on  $\text{Cd}^{2+}$  exposure study (Liu et al. 2012). These antioxidant enzymes, including mRNA levels of *sod*, *cat*, and *gpx1a*, respectively, increased 2.2-, 1.6-, and 3.1-fold after 1 h of  $\text{Cd}^{2+}$  exposure as compared to the control. Our previous study reported that  $\text{Cd}^{2+}$  temporarily accumulates in the gills before being transferred to the digestive organs via the circulatory system or enterohepatic circulation, resulting in lower concentrations of  $\text{Cd}^{2+}$  in the gills than in other organs (Wu et al. 2007). This is the likely cause of the rise in ROS levels in the gills, 1.3- to 1.5-fold after  $\text{Cd}^{2+}$  exposure, which were lower than in the brain.

The uptake of Cd by living cells usually induce ROS to rise, cause oxidative stress (Hsu et al. 2013), and normally leads to cell apoptosis depending on the Cd dose and time of exposure (Chen et al. 2014). However, it should be mediated by some antioxidants. In the present study, ROS levels, respectively, increased 8.0-, 7.1- and 6.2-fold in the brain 1, 3, and 6 h after  $\text{Cd}^{2+}$  exposure, respectively. Therefore, the *sod*, *cat*, and *gpx1a* mRNA levels increased by 1.2-, 16.5-, and 65.6-fold after exposure to  $\text{Cd}^{2+}$  for 1 h, and by 2.4-, 9.2- and 29.5-fold after  $\text{Cd}^{2+}$  exposure for 3 h. We suggest that the LPO levels did not significantly increase since these expressions of antioxidant (*sod*, *cat*, and *gpx1a*) increase in the brain. Observably, these antioxidant expressions also provided a dominant antioxidant system in the brain after  $\text{Cd}^{2+}$  exposure.

Both SOD and CAT directly decrease the toxicity of ROS. SOD is the enzyme which deals with oxygen toxicity. It can remove  $\text{O}_2^-$  through the process of dismutation (super oxide free radical to hydrogen peroxide and oxygen) (An et al. 2010). CAT can reduce  $\text{H}_2\text{O}_2$  into water and oxygen to prevent



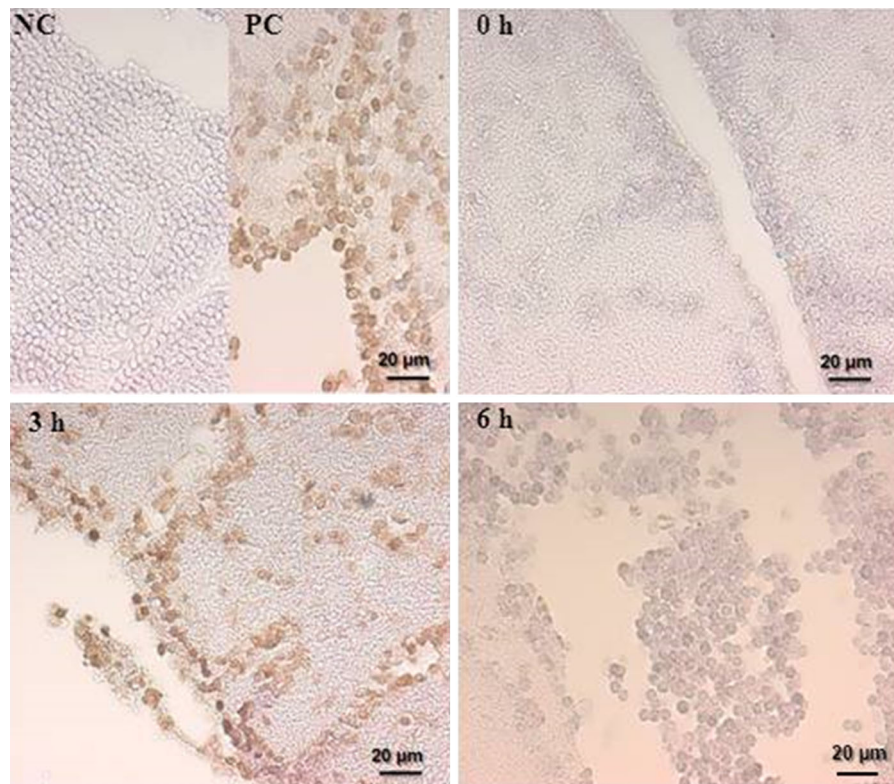


**Fig. 7** Cell apoptosis (brown signals) of zebrafish gills after 0, 3, and 6 h of exposure to  $1.78 \mu\text{M Cd}^{2+}$ . *NC* negative control, *PC* positive control. Magnification  $\times 50$

oxidative stress and maintain cell homeostasis (Kashiwagi et al. 1997). GPx activity is believed to play an important role in cellular antioxidant defense by using glutathione (GSH) as a reducing agent to reduce  $\text{H}_2\text{O}_2$  and various hydroperoxides (Wendel 1980). Similar to previous studies, the present study measured how some anti-oxidative enzymes such as *sod*, *cat*, and *gpx1a* defend the organism against oxidative stress through the  $\text{Cd}^{2+}$  toxicity induction. The three enzyme antioxidants are all interrelated, with the *cat* and *gpx1a* expressions increasing, followed by an increase in *sod* mRNA levels within 3 h of exposure time. Atli and Canli (2010) reported that the stimulation of CAT activity is associated with an effective antioxidant defense system acting against oxidative stress and compensating for the decrease in other antioxidant enzymes, such as SOD and GPx. The present study confirmed the cascade reaction of anti-oxidative system upon  $\text{Cd}^{2+}$  exposure. However, a lack of significant change or decrease in the *cat* mRNA may be attributed to the increase in *gpx1a* and *mt*. In the

gills, *cat* showed no significant change but *gpx1a* and *mt* mRNA levels increased significantly at 3 h after  $\text{Cd}^{2+}$  exposure. In the brain, all antioxidants including *cat*, *sod*, *gpx1a*, *smtB*, and *mt2* mRNA expression increased 3 h following  $\text{Cd}^{2+}$  exposure, but then decreased after 6 h. The LPO level did not significantly change within 6 h after  $\text{Cd}^{2+}$  exposure.

Aside from *SOD*, *CAT*, and *GPx*, *MT* is the best-known antioxidant providing protection against metal toxicity (Choi et al. 2007). The *Cyprinidae* *MT* gene family includes two major groups: *D. rerio mt2* and *MT-A. smtB*; an expressed sequence tag (EST) of *D. rerio* was submitted to GenBank in 1999, and its physiological functions have been gradually explored. Our previous study hypothesized that *mt2* is more important during larval stages, but *smtB* may perform certain physiological functions in the brain of mature zebrafish (Wu et al. 2008a). Moreover, transcripts of *smtB* in the brain increased significantly by 3.5-fold at 1 h after cold shock, which had a higher mRNA expression than that in the gill and liver (Wu et al.



**Fig. 8** Cell apoptosis (brown signals) of zebrafish brains after 0, 3, and 6 h of exposure to 1.78  $\mu\text{M}$   $\text{Cd}^{2+}$ . *NC* negative control, *PC* positive control. Magnification  $\times 50$

2015). The present study showed that the *smtB* mRNA levels were significantly increased, respectively, by 6.5-fold (0.13/0.02) in the gills and 59.4-fold (15.44/0.26) in the brain at 3 h after  $\text{Cd}^{2+}$  exposure. In contrast, the *mt2* mRNA levels expression increased 11-fold in gills and brain after 3 h of  $\text{Cd}^{2+}$  exposure. These results represent that the physiological function of *smtB* might be more important than *mt2* in the brain of the adult zebrafish upon stress.

It is well known that ROS can cause severe damage to cellular macromolecules, especially DNA (Barzilai and Yamamoto 2004). Cell death can be detected using a TUNEL assay to label DNA strand breaks. The present data showed the strongest levels of cell apoptosis in the gills and brain after exposure to  $\text{Cd}^{2+}$  for 3 h, and both tissues showed that the cells were repaired 3–6 h after exposure to  $\text{Cd}^{2+}$  (Figs. 7, 8). Depending on the changes over time, the results of cell apoptosis were found to correspond to the anti-oxidative responses. Furthermore, three enzymatic antioxidant gene expressions (*sod*, *cat*, and *gp1ax*) and

two non-enzymatic antioxidant gene expressions (*mt2* and *smtB*) were confirmed to serve as biomarkers for oxidative stress on fish. The transcripts of the three gene expressions showed a noticeable increase during 1–3 h of  $\text{Cd}^{2+}$  exposure, with an increase in cell apoptosis during that time. In addition, the three genes' transcripts decreased at 6 h, and the apoptosis levels in the brain and gills also decreased. According to these data, we suggested that *mt2* and *smtB* both genes might have two functions upon the fish's exposure to metals: binding metals and defending against oxidative stress. However, under  $\text{Cd}^{2+}$  exposure, *smtB* might play more important roles than *mt2* in defending oxidative stress in brain of adult zebrafish.

## Conclusion

The results of the present investigation show that ROS can be induced by 1.78  $\mu\text{M}$   $\text{Cd}^{2+}$  exposure and do not cause significant oxidative stress in the brain or gill

tissue because many antioxidant genes act to defend against oxidative stress during a short time after Cd<sup>2+</sup> exposure. The brain and gill tissue still exhibit cell apoptosis 3 h after Cd<sup>2+</sup> exposure and recovery after 3–6 h. The present study is the first attempt to compare the antioxidant functions of *mt2* and *smtB* following Cd<sup>2+</sup> exposure between the brain and gills and finds that *smtB* seems to play a more significant antioxidant role in the brain than in the gills, while *mt2* serves a similar function between the brain and gills.

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